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(54) INSULIN DERIVATIVES

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SPECIFICATION NO 1415333

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ceutical preparations containing such insulin derivatives.

It has generally been known that insulins, for example from pigs or oxen, are used for the treatment of diabetes. During prolonged treatment with insulins antibodies are formed which counteract the insulin administered. Owing to the loss in activity by combination with antibodies, the dose of insulin must in these cases be increased.

In German Offenlegungsschrift No. 2,023,447 there are described insulin derivatives having a lower affinity towards insulin-antibodies. The Offenlegungsschrift includes both mono-substituted and di- and tri-substituted derivatives. The mono-substituted derivatives are, however, given prominence as being especially important.

In contradistinction thereto, it has now been found that mono-, di- and tri-carbamoyl-insulins, not hitherto described and especially the di- and tri-carbamoyl-insulins, exhibit a surprisingly high dissociation between their biological activity and their immunological activity. The di- and tri-substituted derivatives are preferred, owing to their being easier to prepare.

The present invention accordingly provides mono-, di- and tri-substituted insulin derivatives in which the terminal amino group of the B-chain (B_1-phenylalanine), or the terminal amino group of the B-chain (B_1-phenylalanine) and the terminal amino group of the A-chain (A_1-glycine), or the terminal amino group of the B-chain (B_1-phenylalanine), the terminal amino group of the A-chain (A_1-glycine) and the B_{29}-lysine group, is or are present in reaction condition and the quantity of reactant used. The best yields of mono- and di-substituted products are obtained at a pH-value of about 7 and not higher than a pH-value of 8. Surprisingly, the dicarbamoyl-derivative is formed almost exclusively when, in the course of the reaction, the pH-value is brought from about 7 to about 5. The formation of the trisubstituted derivative is favoured at a pH-value of 8 to 9.

Adjustment of the pH-value is effected with a buffer substance or with the aid of an autotitrator.

The carbamoylating agent is used in excess. There are thus used for producing the mono-substituted compound 1 to 2 times the molar quantity, and for producing the di- and tri-substituted compounds 150 to 200 times the molar quantity, of carbamoylating agent per molar quantity of insulin.

The reaction is carried out at room temperature or preferably at a slightly raised temperature.

The di- and tri-substituted derivatives can be obtained in a good yield and in a state of high purity. The reaction products can be purified by simple reprecipitation, and do not need to be separated by expensive operations, as does the monosubstituted derivative. The separation of the monosubstituted carbamoyl-insulin is carried out by the methods customarily used in peptide and protein chemistry, for example countercurrent distribution, ion-exchange chromatography, electrophoresis and absorption chromatography.

The present invention accordingly also provides a process for the manufacture of mono-,

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(54) INSULIN DERIVATIVES

(71) We, SCHERING AKTIENGESELLSCHAFT, a Body Corporate organised according to the laws of Germany, of Berlin and Bergkamen, Germany, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention is concerned with new insulin derivatives and with pharmaceutical preparations containing such insulin derivatives.

It has generally been known that insulins, for example from pigs or oxen, are used for the treatment of diabetes. During prolonged treatment with insulins antibodies are formed which counteract the insulin administered. Owing to the loss in activity by combination with antibodies, the dose of insulin must in these cases be increased.

In German Offenlegungsschrift No. 2,023,447 there are described insulin derivatives having a lower affinity towards insulin-antibodies. The Offenlegungsschrift includes both mono-substituted and di- and tri-substituted derivatives. The mono-substituted derivatives are, however, given prominence as being especially important.

In contradistinction thereto, it has now been found that mono-, di- and tri-carbamoyl-insulins, not hitherto described and especially the di- and tri-carbamoyl-insulins, exhibit a surprisingly high dissociation between their biological activity and their immunological activity. The di- and tri-substituted derivatives are preferred, owing to their being easier to prepare.

The present invention accordingly provides mono-, di- and tri-substituted insulin derivatives in which the terminal amino group of the B-chain (B_1-phenylalanine), or the terminal amino group of the B-chain (B_1-phenylalanine) and the terminal amino group of the A-chain (A_1-glycine), or the terminal amino group of the B-chain (B_1-phenylalanine), the terminal amino group of the A-chain (A_1-glycine) and the B_{29}-lysine group, is or are each substituted by a carbamoyl group, and especially the di- and tri-substituted derivatives.

The preparation of the new carbamoyl-derivatives is carried out in a manner known per se by carbamoylating insulin in an aqueous solution. As carbamoylating agents there may be used alkali metal cyanates, for example potassium and sodium cyanates, and ammonium cyanate. Different substitution products can be obtained depending on the pH reaction condition and the quantity of reactant used. The best yields of mono- and di-substituted products are obtained at a pH-value of about 7 and not higher than a pH-value of 8. Surprisingly, the dicarbamoyl-derivative is formed almost exclusively when, in the course of the reaction, the pH-value is brought from about 7 to about 5. The formation of the trisubstituted derivative is favoured at a pH-value of 8 to 9.

Adjustment of the pH-value is effected with a buffer substance or with the aid of an autotitrator.

The carbamoylating agent is used in excess. There are thus used for producing the mono-substituted compound 1 to 2 times the molar quantity, and for producing the di- and tri-substituted compounds 150 to 200 times the molar quantity, of carbamoylating agent per molar quantity of insulin.

The reaction is carried out at room temperature or preferably at a slightly raised temperature.

The di- and tri-substituted derivatives can be obtained in a good yield and in a state of high purity. The reaction products can be purified by simple reprecipitation, and do not need to be separated by expensive operations, as does the monosubstituted derivative. The separation of the monosubstituted carbamoyl-insulin is carried out by the methods customarily used in peptide and protein chemistry, for example countercurrent distribution, ion-exchange chromatography, electrophoresis and absorption chromatography.

The present invention accordingly also provides a process for the manufacture of mono-,

di- and tri-substituted insulin derivatives in which the terminal amino group of the B-chain (B_1-phenylalanine), or the terminal amino group of the B-chain (B_1-phenylalanine) and the terminal amino group of the A-chain (A_1-glycine), or the terminal amino group of the B-chain (B_1-phenylalanine), and the terminal amino group of the A-chain (A_1-glycine) and the B_{29}-lysine group, is or are each substituted by a carbamoyl group, wherein insulin is reacted in an aqueous solution with a carbamoylating agent at a controlled pH-value.

Examination of the blood sugar-lowering action was carried out on rabbits that had been starved for 24 hours. Insulin and its derivatives were injected in an amount of 0.0185 mg per kg. The biological action was determined by measuring the content of blood glucose. All the carbamoyl-derivatives were found, by statistical evaluation (variance analysis), not to be significantly different from insulin, that is to say the derivatives are biologically just as active as insulin.

Examination of the immunological action (capacity for combining with antibodies) was carried out by the radioimmune test of Morgan, Sorenson and Lazarow [cf. Diabetes, 13 (1964) 579]. This showed that the antibody-combining capacity of the di- and tri-substituted carbamoyl-derivatives, which have 7—9% (about 2 insulin units per mg) of the combining capacity of bovine insulin for antibodies active against bovine insulin, was lower than that of all insulin derivatives to our knowledge hitherto described. Moreover, the new carbamoyl-derivatives, as compared with insulin, possess a longer lasting blood sugar-lowering activity.

Owing to their favourable properties the new carbamoyl-derivatives of the present invention are especially well suited for the treatment of diabetes.

The present invention accordingly further provides blood sugar-lowering pharmaceutical preparations having a low capacity for combining with insulin-antibodies, which comprise the new mono-, di- and tri-carbamoyl-insulins in admixture or conjunction with a pharmaceutically suitable carrier.

The pharmaceutical preparations for parenteral use may be, for example, in the form of isotonic or hypotonic solutions containing 40 insulin units of active substance per ml.

Such a pharmaceutical preparation has, for example, the following composition:

40 insulin units of N^{α} - carbamoyl - gly)A_1 - (N^{α} - carbamoyl - phe)B_1 - insulin per ml of an aqueous solution containing 0.16% by weight of sodium acetate, 0.70% by weight of sodium chloride and 0.10% by weight of para-hydroxybenzoic acid methyl ester.

Depot preparations can be made up in the usual manner as a protamine-zinc complex or with "Surfen" (Registered Trade Mark).

The following Examples illustrate the invention:

Example 1

($N^{\prime}$ - Carbamoyl - phe)B_1 - insulin.

600 mg of amorphous zinc-free insulin were dissolved in 60 ml of a 0.1 molar phosphate buffer (pH 7.5), 0.3 ml of a 0.6N-solution of potassium cyanate was added, and the whole was maintained for 18 hours at 30°C. The reaction solution was dialysed against water and then freeze-dried. Yield: 500 mg. In order to separate the ($N^{\prime}$ - carbamoyl - phe)B_1 - insulin there may be used the usual methods of peptide purification, for example ion-exchange chromatography, for example with the use of DEAE-"Sephadex" (Registered Trade Mark) in a 4 to 7 m urea buffer, or countercurrent distribution, for example in the system *n*-butanol (20), methanol (5), water (20), glacial acetic (1). The yield of pure carbamoyl - (phe)B_1 - insulin was 200 mg.

Paper electrophoresis:

Conditions: 2.4 m formic acid/4 m urea, colouring with Pauly reagent. 300 μg were applied. The substance migrated as a unitary band. Its relative migration speed 0.91 (insulin 1.00) corresponds to a monosubstituted insulin.

Example 2

($N^{\prime}$ - carbamoyl - gly)A_1 - ($N^{\prime}$ - carbamoyl - phe)B_1 - insulin.

6 grams of amorphous zinc-free insulin from oxen were dissolved in 600 ml of desalted water, and the solution was adjusted to a pH of 7.2 with a 1N-solution of sodium hydroxide and heated to 30°C. At this temperature 300 ml of a 0.6N-solution of potassium cyanate were added dropwise over a period of about 7 hours. During the same period the pH-value was brought slowly from 7.2 to 5.5 by addition of 1N-acetic acid by means of an autotitrator. After the end of the reaction, the excess of cyanate was decomposed by acidification to pH 2.2 to 2.5. The acid solution was dialysed against water and was then freeze-dried. The yield was 5.3 to 5.5 grams of (N^{α} - carbamoyl - gly)A_1 - ($N^{\prime}$ - carbamoyl - phe)B_1 - insulin. Small amounts of mono- and tri-carbamoyl-insulin can be removed by the usual methods of peptide purification, for example ion-exchange chromatography, counter-current distribution, carrier-free electrophoresis, but advantageously by isoelectric precipitation by dissolving the product at pH 7 and precipitating at pH 4.

Paper electrophoresis:

The substance migrated as a unitary band. Its relative migration speed was 0.76 (insulin: 1.00).

Example 3

(N^{α} - carbamoyl - gly)A_1 - (N^{α} - carbamoyl - phe)B_1 - (N^{ϵ} - carbamoyl - lys)B_{29} - insulin.

6 grams of zinc-free insulin were dissolved in 600 ml of an 8.5 pH-buffer (for example tris-buffer or phosphate buffer) and 300 ml of a 0.6N-solution of potassium cyanate were slowly added dropwise. When the addition had been completed, the excess of cyanate was decomposed by acidification to pH 2.5. The acid solution was dialysed against water and then freeze-dried. The yield was 5.5 grams. Purification is not generally necessary, but it can easily be carried out by the usual methods (see Example 2).

Paper electrophoresis:

The substance migrated as a unitary band. Its relative migration speed was 0.56 (insulin: 1.00) which corresponds to a trisubstituted insulin.

Example 4

The preparation of a sterile neutral injection solution (40 insulin units per ml).

220.0 mg of para-hydroxybenzoic acid methyl ester were dissolved in 205 ml of distilled water at +70°C. After cooling to room temperature, the solution was divided into two approximately equal portions. 325.6 mg of (N^{α} - carbamoyl - gly)A_1 - (N^{α} - carbamoyl - phe)B_1 - insulin were dissolved in one portion and the solution was brought to a pH-value of 7.0 with a 0.1N-solution of sodium hydroxide. 1.540 grams of sodium chloride and 352.0 mg of sodium acetate. $3H_2O$ were dissolved in the second portion, and the solution was also brought to a pH-value of 7.0 with a 0.1N-solution of sodium hydroxide. The two solutions were combined, the pH-value was adjusted to 7.0, and the combined solution was made up to 220 ml with distilled water, filtered under sterile conditions, and introduced under sterile conditions into multiphials of 10 ml capacity.

WHAT WE CLAIM IS:—

1. (N^{α} - Carbamoyl - gly)A_1 - (N^{α} - carbamoyl - phe)B_1 - insulin.
2. (N^{α} - Carbamoyl - gly)A_1 - (N^{α} - carbamoyl - phe)B_1 - (N^{ϵ} - carbamoyl - lys)B_{29} - insulin.
3. (N^{α} - Carbamoyl - phe)B_1 - insulin.
4. A pharmaceutical preparation which comprises the compound claimed in claim 1, in admixture or conjunction with a pharmaceutically suitable carrier.
5. A pharmaceutical preparation which comprises the compound claimed in claim 2, in admixture or conjunction with a pharmaceutically suitable carrier.
6. A pharmaceutical preparation which comprises the compound claimed in claim 3, in admixture or conjunction with a pharmaceutically suitable carrier.
7. A pharmaceutical preparation as claimed in any one of claims 4 to 6, which is in the form of an isotonic solution being suitable for parenteral administration and containing 40 insulin units of active substance per ml.
8. A pharmaceutical preparation as claimed in any one of claims 4 to 6, which is in the form of a hypotonic solution being suitable for parenteral administration and containing 40 insulin units of active substance per ml.
9. A pharmaceutical preparation as claimed in claim 7 or 8, containing 0.16% by weight of sodium acetate, 0.70% by weight of sodium chloride and 0.10% by weight of para-hydroxybenzoic acid methyl ester.
10. A pharmaceutical preparation as claimed in any one of claims 4 to 6, which is in the form of a depot preparation.
11. A pharmaceutical preparation having a composition substantially as described in Example 4 herein.
12. A process for the manufacture of the compound claimed in any one of claims 1 to 3, wherein insulin is reacted in an aqueous solution with a carbamoylating agent at a controlled pH-value.
13. A process as claimed in claim 12, wherein the carbamoylating agent is an alkali metal cyanate or ammonium cyanate.
14. A process as claimed in claim 13, wherein the alkali metal cyanate is potassium or sodium cyanate.
15. A process as claimed in claim 12, conducted substantially as described in any one of Examples 1 to 3 herein.

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